Pharmacological Restriction of Innate Immunity for the Replication and Detection of Human Viral Pathogens

A Senior Honors Thesis

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Abstract:

The focus of this study is to explore the immunosuppressive effect of the chemotherapy drug fludarabine and its application to viral cultivation. Fludarabine is mainly used in the treatment of chronic lymphocytic leukemia. It is a purine analog and inhibits DNA synthesis by interfering with ribonucleotide reductase and DNA polymerase. Some studies also show that fludarabine induces severe and prolonged immunosuppresion; such effects often increase the risk of opportunistic infections. This study explores whether fludarabine may enhance viral infection *in vitro* and aid the process of cultivating viruses that are previously uncultivable, such as Norovirus. The immediate goal was to develop a standardized procedure to treat cell lines with fludarabine, suppress innate immunity while retaining cell viability, and enhance viral infections. Carcinomic human alveolar basal epithelial cells (A549) were used to first test for cytotoxicity of fludarabine, and an optimal concentration to use that would still retain cell viability. After obtaining an optimum drug concentration, qualitative and quantitative methods were used to determine whether fludarabine treatment enhances viral infectivity; bovine enterovirus-1 (BEV-1), a single stranded RNA virus having positive polarity, was used for the test. Treatment exposure time, drug treatment method (pre-treatment or continuous exposure), and viral samples concentrations were all testable variables. The results indicated that fludarabine treated cells exhibited greater cytotoxicity than non-treated cells when infected with BEV-1; however, the difference in viral yields between drug treated and non-treated cells were inconclusive and required further experimentations. The study also discusses potential methods to improve the fludarabine treatment protocol.

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Abbreviations Glossary: [1,3,20]

- *CARD:* Caspase recruitment domain
  A common interaction motif that mediates protein-protein interaction.

- *Fv:* Friend-virus susceptibility protein
  A murine anti-viral protein that suppresses antibody expression against Friend virus infection.

- GCN2: eukaryotic translation initiation factor 2 alpha kinase 4
  A eukaryotic kinase that phosphorylates the alpha subunit of translation initiation factor-2, which down-regulates protein synthesis in response to cellular stress such as viral infection.

- *IFN:* Interferon
  Cytokines released by lymphocytes in response to pathogenic invasion, and also serve as a mean of cellular communication.

- *iKK:* iKβ kinase
  An enzyme that activates NF-κB in response to an inflammation.

- *IPS-1:* Interferon-beta promoter stimulator 1
  An adaptor molecule that transduces inflammatory signals to transcription factors or regulatory factors. It involves an MDA5 or RIG-1-mediated immune responses.

- *IRF:* Interferon regulatory transcription factor
  Protein that regulates the transcription of interferon-encoding genes.

- *JAK:* Janus kinase
  Enzyme that belongs to tyrosine kinases family, which serves as a binding site of STAT molecules and activates STAT through phosphorylation.

- *MDA-5:* Melanoma differentiation-association gene-5 protein
  An RNA helicase that recognizes viral RNA and passes down signal through signal transduction.

- *Mx:* Myxovirus resistant interferon-inducible protein
  A murine protein which induces certain antiviral responses, primarily against *Influenza* virus.

- *NAP-1:* Neutrophil activating peptide-1
  A cytokine that signals for neutrophil activation upon interacting with the cytokine receptors located on neutrophils.

- *NF-κB*: Nuclear factor kappa-light-chain-enhancer of activated B cells
  A transcription factor responsible for transcription of many genes related to immune responses.

- *NLRs:* Nod-like receptors
  A family of proteins that locate in the cytoplasm and serve as regulators of immune responses. Many NLRs serve as pattern-recognition receptors.

- *PAMPs:* Pathogen-associated molecular patterns
  Molecular features that are recognized by components of innate immunity, specifically pattern-recognition receptors. PAMPs may trigger immune responses.

- *PKR:* Protein kinase R
  A eukaryotic protein that displays anti-viral activity by binding to double-stranded RNA. PKR production is triggered by interferons.

- *PRRs:* Pattern-recognition receptors
  Components of innate immunity that recognize pathogenic molecular patterns, or PAMPs, and trigger appropriate immune responses.

- *RAG:* Recombination activating gene
  Gene which encodes for RAG-1 and RAG-2 proteins, which are critical for initiating DNA recombination for the proper expression of antigenic receptors and maturation of B and T lymphocytes.

- *RIG-I:* Retinoic acid-inducible gene 1 protein
  An RNA helicase that recognizes viral RNA and passes down a signal through signal transduction.

- *SINTBAD:* Similar to NAP1 TBK1 adaptor,
  A protein that serves as signal transducing activator. The name suggests its sharing of the binding domain with NAP-1 and TBK proteins.

- *STAT-1:* Signal Transducers and Activator of Transcription protein-1
  A protein that serves as signaling molecule and transcription factor in response to cytokine receptor's binding to cytokines. It belongs to a family of STAT molecules.

- *TANK:* TRAF family member-associated NF-κB activator
  A regulatory protein that locates in the later stage of signal transduction pathway and is responsible for regulating the transcription of immune response proteins.

- *TBK:* TANK-binding kinase
  A family of proteins that serve as transcription factors regulator, similar to iKβ kinase.

- *TLRs:* Toll-like receptors
  Receptor molecules that locate on cell membrane or endosomal membrane and serve as pattern recognition receptors.

- *TRAF-3:* Tumor necrosis factor receptor associated factor-3
  The protein that mediates signal transduction of responding to antigenic invasion and activating immune responses.

- *TRIM-5α:* Tripartite motif-containing 5-α protein
  A protein found in most primates and is found responsible for innate immune responses against retroviruses.

Introduction:

- *Diversity of Viruses and Their Recognition by cells -- Overview*

Innate immunity is the non-specific response carried out by hosts against molecular invasion. For humans, innate immunity serves as the first line of defense, as compared to the acquired immunity which responds to antigens once they have breached the innate immunity [1]. Despite being non-specific, innate immunity utilizes vastly diverse mechanisms against antigens, such as physical or chemical barriers like skin or mucous membrane to prevent entry, phagocytes or natural killer cells to physically isolate and eradicate foreign microbes, blood proteins like complement system or other mediators to damage microbes or enhance other immune responses, or protein regulators like cytokines to regulate many aspects of innate and acquired immunities [1]. While technological advancement and researchers' effort have contributed many breakthroughs in understanding immunology, innate immunity against viral infection remains as an area that many questions are yet unanswered.

For a host cell to initiate immunity against a viral infection, it must first be able to recognize the virus as foreign. Viral recognition mechanisms are related to the genetic characteristics, infecting methods, viral components, and the identity of the invading virus [1,3]. Firstly, genetic characteristics refer to the genetic makeup of a virus, which can be one of the four variations: single-stranded DNA, double-stranded DNA, single-stranded RNA, or double-stranded RNA [28]; ssRNA viruses are further categorized into sense and anti-sense depending on their RNA sequence being identical or complementary to its encoding proteins. Secondly, infection methods refer to how the viral genome enters the host cell. Most viral genomes gain entry into a host through either fusion of the cell membrane, which then locate within the cytosol, or through receptor-mediated uptake (phagocytosis), which then locate within the endosome [3].

Thirdly, different viral components may trigger different responses of the infected host. For instance, presence of viral proteins in cytosol could trigger cytosolic restriction factors such as tripartite motif-containing 5-α protein (TRIM-5α) in human, or friend-virus susceptibility protein (Fv) and myxovirus resistant interferon-inducible protein (Mx) in mice [15,22,28], while presence of viral nucleic acids could elicit elF2α-phosphorylating kinases such as protein kinase R (PKR) or eukaryotic translation initiation factor 2 alpha kinase 4 (GCN2) [15,22]. Lastly, different species of viruses that have similar genetic makeup could trigger different responses by the host. For example, positive-sense single-stranded RNA ((+)ssRNA) viruses, Picornaviruses and Caliciviruses are recognized by melanoma differentiation-association gene 5 (MDA-5), Influenza viruses are recognized by retinoic acid inducible gene-I (RIG-I), and Paramyxoviruses, West Nile viruses, and Dengue viruses are recognized by both [9,10,14]. These four factors depict the vast diversity of viruses as well as the potential difficulty of studying viral recognition due to such diversity.

- *Pattern-Recognition Receptors (PRRs) Involved with Pathogen Recognition*

PRR is a general term to describe a group of protein receptors expressed by host cells to recognize pathogen-associated molecular patterns (PAMPs) [1]. The structures of PRRs are conserved, i.e. their PAMPS recognition abilities are not species-specific (but rather patterns-specific), and therefore PRRs belongs to part of the innate immunity [1]. The main function of PRRs, in simple terms, is to recognize microbial molecules that are not commonly expressed in self. Scientists discovered that PRRs have existed much earlier than adaptive immunity, and in some occasions serves as the only known form of immune system in less evolved organisms [1,23]. In more developed organisms, their PRR system contains a certain level of complexity to

allow recognition of many microbial molecules, therefore triggering appropriate immune responses [1].

One of the most important PRRs is toll-like receptors (TLRs). TLRs are evolutionarily conserved and expressed on a variety of cell types, including macrophages, dendritic cells, neutrophils, mucosal epithelial cells, and endothelial cells [26]. Currently there are eleven types of human TLRs identified, and they can be categorized into two groups: one group of TLRs is expressed on plasma membranes to recognize extracellular PAMPS; another group of TLRs is expressed on endosomal membranes to recognize intra-endosomal PAMPS [1,26]. The structure of TLRs are composed generally of two portions: a recognition portion containing leucine-rich repeat motifs and cystein-rich flanking motif that recognizes PAMPs, and a signaling portion containing a Toll/IL-1 receptor homology domain that transduces the signal of PAMPs detection to other parts of the cell [26]. The specificity of TLR binding sites to different types of PAMP ligands is observed, such as TLR2 recognizing bacterial peptidoglycan and endotoxin or TLR5 recognizing bacterial flagellin; however, the structural analysis of the binding sites carrying out such specificities is not yet well-understood [1,20,26]. Once the signal of PAMPs recognition is transduced, the cell reacts by secreting cytokines for inflammatory or anti-viral responses, secreting chemokines for phagocyte recruitment, releasing costimulatory molecules to activate adaptive immune cells, or expressing adhesion molecules to enhance phagocytosis [16,26].

Besides TLRs, other plasma membrand and cytoplasmic PRRs are also expressed on various cell types. These PRRs recognize PAMPS using different structures other than those of TLRs, but the signal transduction mechanisms are similar, including promotion of inflammatory cytokines or enhancement of phagocytosis [1]. There are five main non-TLR receptors studied by immunologists. First, C-type lectins are expressed mainly on phagocytes such as macrophages,

dendritic cells, or other leukocytes [1]. They recognize the carbohydrate structures expressed on microbial cell walls but not mammalian cells. Second, scavenger receptors recognize oxidized lipoproteins and promote phagocytosis of these molecules and are expressed primarily on phagocytes [1]. Third, N-formyl Met-Leu-Phe receptors are expressed by neutrophils and macrophages, and they recognize short peptide sequences containing N-formylmethionyl residue [1]. Since very few mammalian cells contain such peptides while all bacterial proteins are initiated by N-formylmethione, these receptors allow phagocytes to effectively recognize and phagocytose bacterial. Fourth, Nod-like receptors (NLRs) are expressed on cytoplasmic membranes of many cells and recognize derivatives of peptidoglycan, common for bacterial cell walls (Nod = nucleotide-binding oligomerization domain) [1]. Lastly, caspase activation and recruitment domain (CARD)-containing proteins are located in the cytoplasm and recognize viral RNA. CARD-containing proteins are discussed more in detail here since they relate to the recognition of (+)ssRNA viruses, which is central to this research study [1,14,18,27].

CARD-containing proteins include retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-association gene 5 (MDA5), both are targets of many research groups studying anti-viral innate immunity [1,18]. Studies show that both RIG-I and MDA5 play an essential role for RNA viruses recognition [14,18,27]. While many TLRs also exhibit viral genome recognizing activity, TLRs recognize viral genomes that are present extracellularly or intra-endosomally due to their physical locations on plasma and endosomal membranes (they also recognize viral DNA and RNA, while RIG-I and MDA5 recognize only viral RNA) [12,20]. For viral RNA presents in cytoplasm, RIG-I and MDA5 are responsible for its recognition [1,14,18,27]. When a single-stranded RNA enters the cytoplasm during viral infection, it begins replication and transcription processes, which the CARD domain of RIG-I or MDA5 recognizes

the post-transcribed 5'-triphosphate ssRNA or post-replication dsRNA (which do not exist in mammalian cells) and binds to it [18,27]. After binding, activated RIG-I or MDA5 interact with interferon-beta promoter stimulator 1 (IPS-1) on mitochondria, with the assistance of TNF receptor associated factor-3 (TRAF-3) activation, thus up-regulating an anti-viral response pathway [27]. Then, activated TRAF-3 recruits a series of signal transducing activators, including TRAF family member-associated NFκB activator (TANK), neutrophil activating peptide-1 (NAP-1), and similar to NAP1 TBK1 adaptor (SINTBAD). Once the activators are recruited, kinases like TANK-binding kinase (TBK) and iKβ kinase (iKK) are recruited, and phosphorylate interferon regulatory transcription factors (IRF-3 or IRF-7) [18,27]. Lastly, phosphorylated IRFs translocate in the nucleus and induce transcription of Type-I interferons (IFN) encoding genes, thus the production of Type-I IFN is activated [25,27]. Illustration of the (+)ssRNA viral recognition pathway is shown in Graph 5-(a).

- *Antiviral Activity – Jak-STAT Pathway Induced by Type-I IFN*

Type-I IFNs, particularly IFN-α and IFN-β, can trigger a cell's antiviral activity [1,19,25]. During viral infection with ssRNA viruses, the infected cells recognize viral genomes and release Type-I IFN using mechanisms stated previously. Type-I IFN induces an antiviral state in near-by cells using a Jak-STAT pathway [1,2]. Firstly, extracellular Type-I IFNs bind with IFN receptors expressed on cell surfaces. Within the cell, IFN receptors are initially bound by inactivated Janus kinase (JAK); upon Type-I IFNs' binding with the receptors, JAK becomes activated [1,2]. Activated JAK, which displays high phosphorylating activity, produces phosphotyrosine residues which serve as a binding site for Signal Transducers and Activator of Transcription (STAT) proteins through the creation of SH2 domains. When a STAT protein binds to the complex, it is

also phosphorylated by JAK and thus creates SH2 domains (thus binding sites) for another STAT protein. Depending on the type of STAT, homodimers or heterodimer of STATS are required for proper activation and subsequent signal transduction. It has been shown that Type I interferons induce STAT-1/STAT-2 heterodimer formation [1,2]. Activated STATs dimers translocate to the nucleus and serve as transcription activators on the target cytokine-responsive genes by binding to STAT-binding sequences in the promoter region. In terms of innate antiviral activity, STAT-1 is the key for proper type-I IFN response and innate immunity activation; innate antiviral activity includes inhibition of viral replication or infected cell's proliferation [3,19,22] Studies show that STAT-1 impaired hosts (through genetic knock-out or natural immunodisorders) are susceptible to bacterial and viral infections as these individuals' innate immunities are defective [1,6]. Type I IFN-induced pathway is shown in Graph 5-(b).

- *Anti-interferon Drug – Fludarabine*

Fludarabine is a drug used for treatment of hematological malignancy, particularly chronic lymphocytic leukemia [17]. Its purine analog structure interferes with activities of DNA polymerases and ribonucleotide reductase, which in turn affects the process of DNA synthesis; such a pharmacological effect enables fludarabine to be active against both dividing and resting cells [17]. In addition, the structure of fludarabine resembles a fluorinated nucleotide analog of an antiviral agent vidarabine, 9-b-D-arabinofuranosyladenine, which displays antiviral activity against double-stranded DNA viruses [17].

Usage of fludarabine is deemed effective against certain hematological cancers; in addition, its immunosuppressive effect has been studied as well [29]. The drug description of fludarabine states that the chemical would induce immunodeficiency; however, the precise

mechanism of such immunosuppression is not yet well understood [29]. One study in 1999 suggested that fludarabine interferes with Signal Transducers and Activators of Transcription-1 (STAT-1) signaling, thus inducing innate immunosuppression [8]. The researchers in the study observed that fludarabine caused a long-lasting immunosuppression effect *in vivo* and made a hypothesis that the suppression was a result of STAT repression [8]. Through *in vitro* testing, the researchers concluded that fludarabine induces loss of STAT-1 proteins in treated cells.

Suppression of STAT-1 protein expression has been shown to be relevant to increases in viral infections [1,6,13]. One study showed that STAT-1 knocked-out mice are susceptible to viral infections [6]; researchers stated that knocked-out mice "failed to thrive and were extremely susceptible to viral disease," and "cells and tissues from STAT-1 (-/-) mice were unresponsive to interferon, but remained responsive to all other cytokines tested" [6]. Another similar study showed that STAT-1/RAG knocked-out mice were susceptible to viral infections (RAG, recombination activating gene, relates to cell-mediated immunity) [13]. This particular study utilized murine norovirus-1 (MNV-1) for testing, and the researchers found that "a STAT-1 dependent innate immune response was sufficient to prevent lethal MNV-1 infections" [13].

- *Linking Paradigm Pieces Together: Hypothesis and Projections of the Study*

The aim of this study was to use bovine enterovirus type I (BEV-1) as a model system for Norovirus and test whether cells that are exposed to fludarabine would be more susceptible for (+)ss-RNA viral infections. As mentioned previously, BEV-1 and Norovirus display much genetic similarity and are both recognized by cells through the MDA5 pathway [21,22]; another study also showed that MDA5 is essential for recognizing norovirus [21]. Relying on the similarities of Picornaviruses (BEV-1) and Caliciviruses (Norovirus), BEV-1 should be a reliable

model system. Moreover, if fludarabine treatment successfully suppresses STAT-1 expression in cells, the JAK-STAT1 pathway would be disrupted, thus the infected cells would not be able to signal neighboring cells to trigger anti-viral responses [8]. In other words, even though if MDA5 successfully recognizes the presence of BEV-1 and triggers the production of Type I interferon, the interferon would not be able to signal anti-viral responses due to JAK-STAT1 disruption. It is still unclear whether STAT-1 suppression would influence Type I interferon through downstream regulation or not, but previous literatures did suggested the connection between viral infectivity and STAT-1 suppression, and this study will further explore such a connection [13].

The hypothesis for this study is that fludarabine treatment will reduce a cell's innate immunity against infections from (+)ssRNA viruses. A confirmation of the hypothesis could lead to the development of a protocol of enhancing viral yields by first treating cells with fludarabine. Since Norovirus is among the group of viruses that are difficult to cultivate in a laboratory setting [4,5,11,24], suppression of a cell's innate immunity against such viruses may enhance viral infectivity and succeed in cultivation. Pharmacological approach of immunosuppression is also more economical, than genetically engineer cell lines with knocked-out immune system pathways, due to the relatively lower cost and non-specific effect. In addition, Norovirus cultivation may allow vaccine production against such viruses, as well as further immunological studies about these viruses, such as determining the numbers of Norovirus serotypes. Currently, Norovirus samples can only be obtained through clinical isolates. The establishment of an *in vitro* fludarabine-treatment protocol could provide a way to determine the presence of this infectious agent in samples taken from the environment or patients, thus allowing prevention of norovirus transmission, nosocomial infections, and clean up of norovirus contamination.

Materials and Methods:

Growth Medium:

Opti-MEM with L-glutamine and phenol red indicator (Invitrogen, Carlsbad, CA) was chosen as the cell culture medium. Amount of supplementation of fetal calf serum (FCS) varied depending on the experimental design: 1% (v/v) or 2% (v/v) for cell maintenance; 8% (v/v) for cell growth; 10% (v/v) for cell enrichment. Also, 50 μg/ml of gentamicin was added to the medium to prevent bacterial contamination.

Cell Culture:

The cell line chosen for experiments was A549, which are carcinogenic human alveolar basal epithelial cells. The cells were stored in sealed glass vials at -70℃ in a liquid nitrogen storage system with dimethyl sulfoxide present in a storage medium. Prior to experimentation, the cells were removed from vials and planted into T-25 tissue culture flasks with 15 ml of 1X Opti-MEM (10% FCS, 50 μg/ml gentamicin, 9mM $NaHCO_3$) as enrichment medium and incubated at 37 ℃ in the presence of 5% CO_2. Passage of cells was carried out weekly to ensure cell vitality and prevent cellular overgrowth. After weekly cell growth, the growth medium was removed from flasks, cultures were washed with serum-free 1X HBSS (Hank's Balanced Salt Solution, Sigma, St. Louis, MO, and 50 μg/ml gentamicin), digested with 1 ml of 0.25% trypsin, and ~1/10 of the cell suspensions were planted into a new T-25 flask with 6 ml of growth medium, 1X Opti-MEM (8% FCS, 50 μg/ml gentamicin, 9mM $NaHCO_3$), to make a 1:10 passage.

Fludarabine Treatment:

A supply of FLUDARA (for injection) was graciously provided by Bayer HealthCare Pharmaceuticals. FLUDARA was packaged as 2 ml per vial, and each vial contained 50 mg of fludarabine phosphate, 50 mg of mannitol, and NaOH to adjust pH to 7.7. Using the molecular weight of fludarabine phosphate, 365.212 g/mol, the concentration of fludarabine phosphate in each FLUDARA sample was 68.45 μM/ml.

A continuous fludarabine exposure method and pre-treatment fludarabine method were both tested to see which method was suitable for an experimental design; the desirable method should cause minimal cell damage after treatment. To observe fludarabine's toxicity, A549 cells planted in 24-well plates were treated with 1.0 ml of undiluted FLUDARA (which contained 68.45 μmol of fludarabine phosphate) and observed daily; the cells displayed cytopathology one day after initial treatment. Then, to determine the dosage of FLUDARA that would induce minimal drug cytopathology, ten-fold and five-fold serial dilutions of FLUDARA were prepared using 1X Opti-MEM (1% FCS, 50 μg/ml gentamicin, 9mM $NaHCO_3$) as diluent, and each drug dilution was used to treat A549 cells growing in 24-well plates. For the continuous drug exposure method, 2.0 ml of a FLUDARA dilution was added to each well and incubated at 37 ℃. For the pre-treatment drug method, 2.0 ml of a FLUDARA dilution was added to each well and incubated at 37 ℃, and after 24 hours the FLUDARA-containing medium was removed and replaced with 2.0 ml of 1X Opti-MEM (1% FCS, 50 μg/ml gentamicin, 9mM $NaHCO_3$), then incubated at 37 ℃. FLUDARA-treated cells in 24-well plates were observed daily and scored visually for drug cytotoxicity. The remaining experimental designs utilized the 24-hour, 0.033 μM/ml FLUDARA pre-treatment method since such method and concentration demonstrated least drug-induced cytotoxicity. The goal of FLUDARA treatment was to suppress innate

immunity of cells and enhance viral infectivity; pretreatment of cells with a low dose of FLUDARA could minimize the chance of decreases in viral infectivity due to drug-induced toxicity.

Drug cytotoxicity was determined by visually comparing the FLUDARA-treated cells in experimental wells to those without treatment in control wells. When pre-treated cells began to show shrinkage due to drug, the numbers of shrunk cells were estimated and recorded as a percentage and compared to untreated cells (growth of untreated cells represented 0% drug cytotoxicity). A numerical rating system was utilized to represent the percentage of cell shrinkage: + for less than 10%, 1 for 25%, 2 for 50%, 3 for 75%, and 4 for 100%.

Bovine Enterovirus Type I (BEV-1):

BEV-1 samples used in the experimentation were maintained in the laboratory and were initially obtained from the National Institute of Health. Viral samples were stored in sealed glass vials at -70℃. Upon usage, the samples were gradually thawed on an ice bath to minimize viral protein denaturation. Prior to inoculation, viral samples were made into appropriate dilutions, depending on experimental design, using 1X Opti-MEM (2% FCS); all viral dilutions were kept on ice until the viral inoculation step.

Qualitative Viral Analysis – Visual Inspection for Viral Cytopathology:

Twenty Thousand of A549 cells were planted in 12-well plates, with 2.0 ml of 1X Opti-MEM (8% FCS) as growth medium, and incubated at 37℃. When the cells reached ~90% confluence (after two to three days of incubation), the medium was removed from all wells, and 2.0 ml of 0.033 μM/ml FLUDARA with 1X Opti-MEM (2% FCS) as diluent was added to six

wells for pre-treatment; the other six wells served as non-treated controls and 2.0 ml of 1X Opti-MEM (2% FCS) was used. The plates continued to incubate at 37℃ for twenty-four hours and were then ready for BEV-1 inoculation. First, five ten-fold serial dilutions of a thawed BEV-1 sample were prepared using 1X Opti-MEM (1% FCS) as diluent. Then, the medium in the 12-well plates was removed, and each well was washed with serum-free 1X HBSS twice. After washing, five wells from treated and un-treated control wells were inoculated with different 200 µl of a BEV-1 dilution, and 200 µl of 1X Opti-MEM (1% FCS) was added to the remaining two virus-free control wells. A time period for viral absorption was done to ensure infection, with the plates being incubated at 37℃ for two hours with agitation every 20 minutes. After two hours, 1.0 ml of 1X Opti-MEM (2% FCS) was added to each well, and plates were returned to a 37 ℃ + 5% CO_2 incubator and observed daily for cytotoxicity. Once the infection process and infected cell observation was done, the content of the wells were collected with pipettes and saved in sealed test tubes in -70℃ freezer until quantitative analysis.

Quantitative Analysis – Immunofluorescence Assay for Detection of Viral Infected Cells: [7]

Ten thousand A549 cells were planted onto a round coverslip (10 mm in diameter) within a 12 x 32 mm borosilicate shell vial, with 1.0 ml 1X Opti-MEM (8% FCS) as growth medium, and incubated at 37℃; four vials were prepared per trial. Size 0 green rubber stoppers were used to seal the vials to prevent excessive evaporation. When cells reached ~90% confluence, growth medium was removed from all vials, cells were washed once with serum-free 1X EBSS, and samples collected from qualitative analysis were used as inoculums to infect these cells. Two hundred µl of BEV-1 (undiluted or 10^{-1} dilution, diluted with Opti-MEM 1% FCS) were added to each vial, and the vials were placed back in 37℃ incubator. Throughout the infection period, the

vials were agitated every 20 minutes; two vials (infection process) were stopped after 5 hours, and the other two vials were stopped after 7 hours. At the end of the vial infection process (after either 5 or 7 hours), medium containing BEV-1 was removed from the culture vial, the cells were washed once with 1X HBSS, and 2 ml of -20℃ acetone (100% v/v) was added to the vial for 15 minutes of cell fixation. Then, acetone was drawn off, and 1.0 ml of 1X Dulbecco's PBS (Irvine Scientific, Santa Ana, CA) was added to each vial as storage medium. Vial samples were stored at -70℃ until viral detection occurred by indirect immune fluorescences.

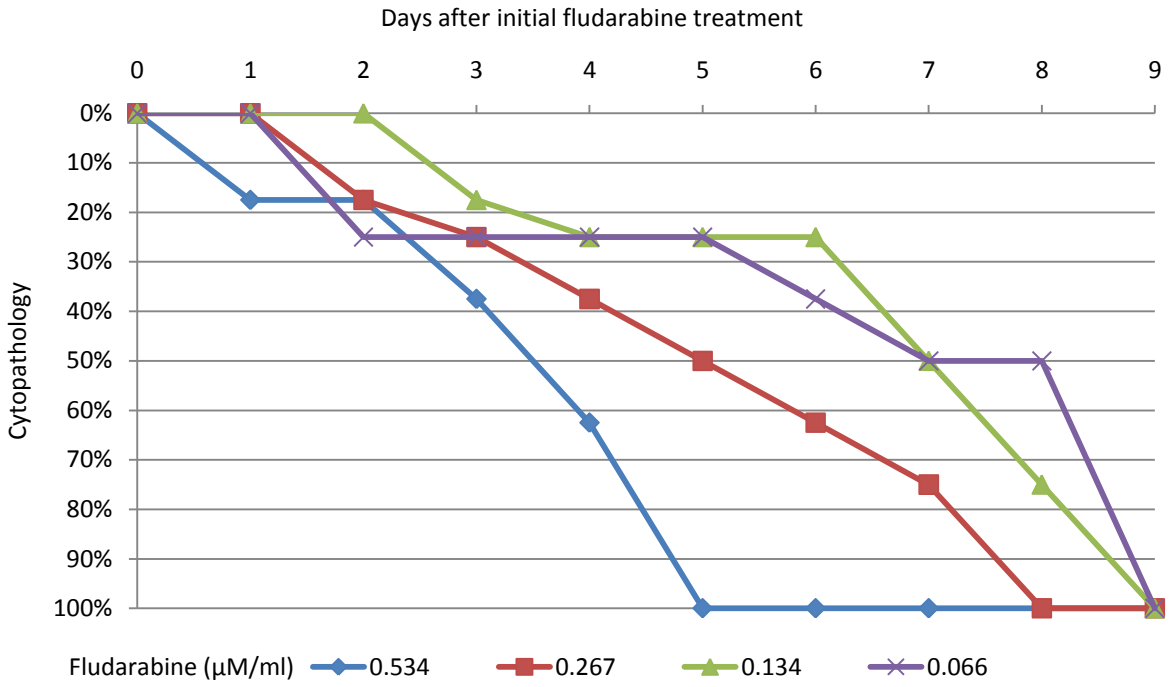
To detect the infected cells for the presence of virus, an immunofluorescence protocol was carried out by first thawing the vial samples and removal of the storage medium [7]. Then, 200 µl of 1:40 diluted anti-peptide antibody (7A) to BEV-1 group antigen was added to each vial; 7A antibody (prepared and supplied by Dr. John Hughes at OSU) served as a primary antibody and was diluted with 1X Dulbecco's PBS. The infected vials were incubated at 4℃ with constant shaking for twenty-four hours, and the primary antibody was then removed, followed by one wash with 1X HBSS. Then, 200 µl of 1:80 diluted Goat anti-Rabbit IgG, FITC (in 50% glycerol) was added to each vial (provided by Dr. Bishop at OSU). The goat anti-rabbit IgG served as secondary antibody and was diluted with 1X Dulbecco's PBS as well. The vial samples were incubated at 37℃ on a tilt shaker (low speed; ~6 tilting cycles per minute) for at least five hours; vials were covered with aluminum foil to minimize fluorophore degradation since the fluorophore is light sensitive. After incubation, secondary antibody was removed from the vials and one washing was done with 1X HBSS. Then, 300 µl of Evan's Blue was added to each vial to counter-stain the cells and allow easier fluorescence detection of viral infected cells. Counter-staining continued for two minutes, followed by two washing steps with dH_2O. Lastly, infected coverslips were removed from each vial, placed inverted (cell monolayer facing down) on a

microscope slide, and observed with a fluorescent microscope under 20X magnification. Three photos were taken from different random locations on a single coverslip, and the data were collected using these photos. If not immediately observed, coverslips were stored in the dark to avoid fluorophore degredation.

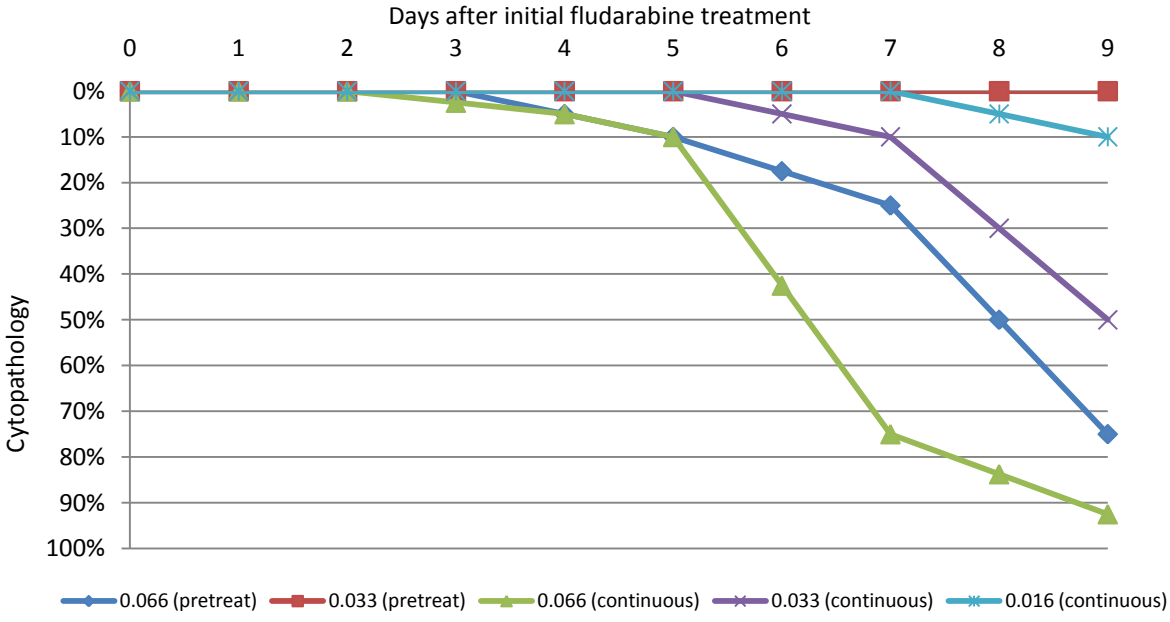
Results:

The results of FLUDARA toxicity test are shown in Graphs 1 and 2. In graph 1, cells under continuous exposure displayed different levels of cytotoxicity depending on the FLUDARA concentrations. FLUDARA at 0.534 μM/ml caused cells to reach 100% cytotoxicity five days after the initial exposure, while 0.134 and 0.066 μM/ml of the chemical caused the same effect on the ninth day after initial exposure. Also, the higher concentration of FLUDARA began to cause cytotoxicity on the first day after exposure, and the rate of cell death was rapid; lower concentration of FLUDARA induced cellular cytotoxicity on the first or second day post-exposure, and the rate of cell death was slower. In graph 2, the results of two methods of FLUDARA treatment, continuous exposure and 48-hour pre-tretment, are presented. Since the first toxicity experiment showed that lower concentrations of FLUDARA caused minimal cytotoxicity for cells, when testing the two treatment methods, low concentrations of FLUDARA (0.066, 0.033, and 0.016 μM/ml) were used. Graph 2 shows that pre-treatment with 0.033 μM/ml FLUDARA caused the least cytotoxicity throughout the nine days of testing, even lower than continuous exposure with 0.016 μM/ml of FLUDARA. In addition, when comparing the results of drug pretreatment and continuous drug exposure using 0.033 μM/ml FLUDARA, pretreatment also yielded lower cytotoxicity than continuous exposure, which began to show effects on cells five days after the initial exposure. Therefore, 0.033 μM/ml was chosen as the suitable

FLUDARA concentration for later experimentation because it was the minimum dosage that caused least cytotoxicity on cells yet could still affect cellular functions.

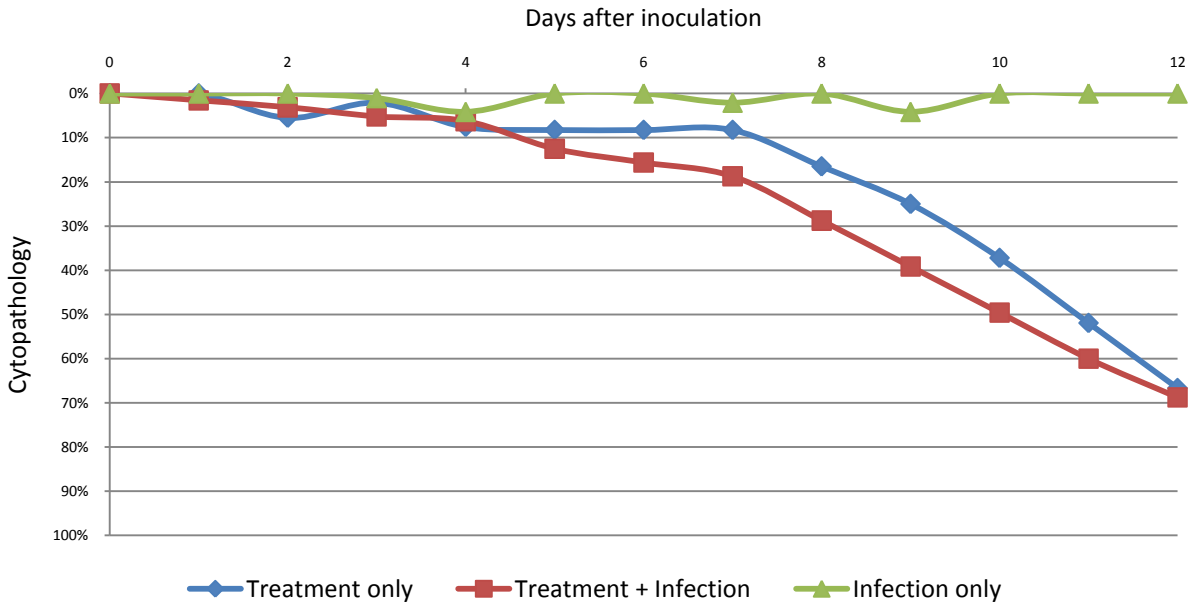


Graph 1: level of cytopathology exhibited by A549 cells with different fludarabine concentrations. The cells were continuously exposed to these concentrations.

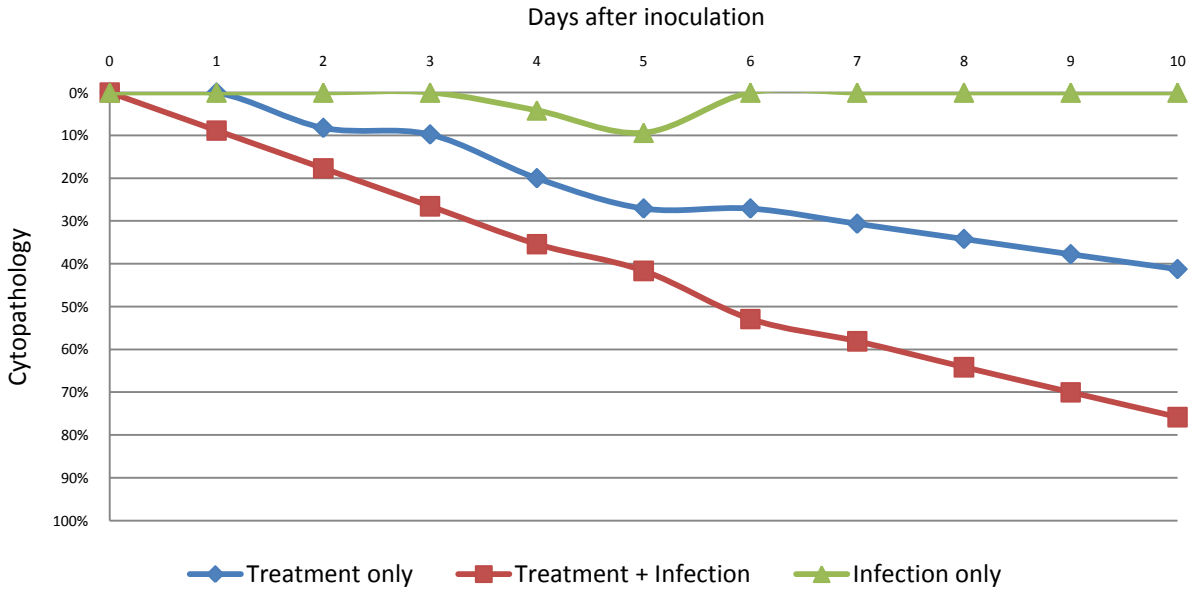


Graph 2: level of cytopathology exhibited by A549 cells with different concentrations of fludarabine. Two methods (pre-treatment and continuous exposure) are shown.

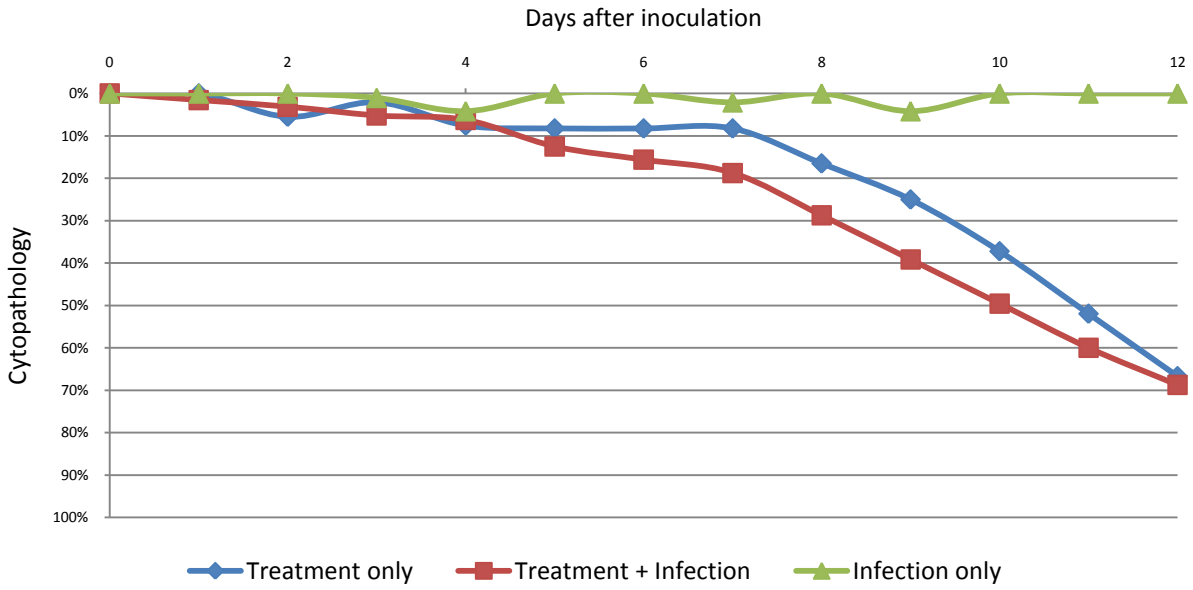
Qualitative viral analysis was done in order to verify whether A549 cells that were exposed to fludarabine would show less resistance to BEV-1 infection than cells that were not treated. Graph 3 shows results from four 24-well plates. Between cells that were treated and infected (T+I) and treated only (T only), T+I cells exhibited a greater death rate (cytotoxicity): graphs 3-(a) and 3-(c) begin to show difference between T+I and T only cells on day five. Also, graph 3-(b) shows that one day after viral inoculation the T+I cells began to show cytopathology; T only cells began to show cytopathology on the second day post-inoculation. Also, graphs 3-(a), (b), and (c) show results for infected only (I only) cells. The amounts of viruses used for inoculation were sufficient for successful infection but not too high to overwhelm the cell culture within short periods of time. The three graphs show that I only cells began to display cytopathology due to viral infection on the third to fifth day after inoculation; decrease of cytopathology afterwards could be due to the cells' growth rate surpassed the rate of infection. Even though I only cells' viral cytopathology remained closely to 0%, the difference in level of cell cytopathology between T only and T+I cells was observed, indicating that fludarabine treatment appeared to enhance BEV-1 infectivity even with low amounts of viruses present. Graph 3-(d) shows similar results as 3-(a), (b), and (c), but the results of T only cells are not shown. Also, notice that negative control cells, i.e. non-fludarabine treated and non-BEV-1-infected, were not included on the graphs. As stated in the Materials and Methods section, the qualitative analysis data were scored visually by comparing cell growth of experimental cells to that of controlled cells; therefore, the cytotoxicity of negative control cells was essentially 0% throughout the observation period.



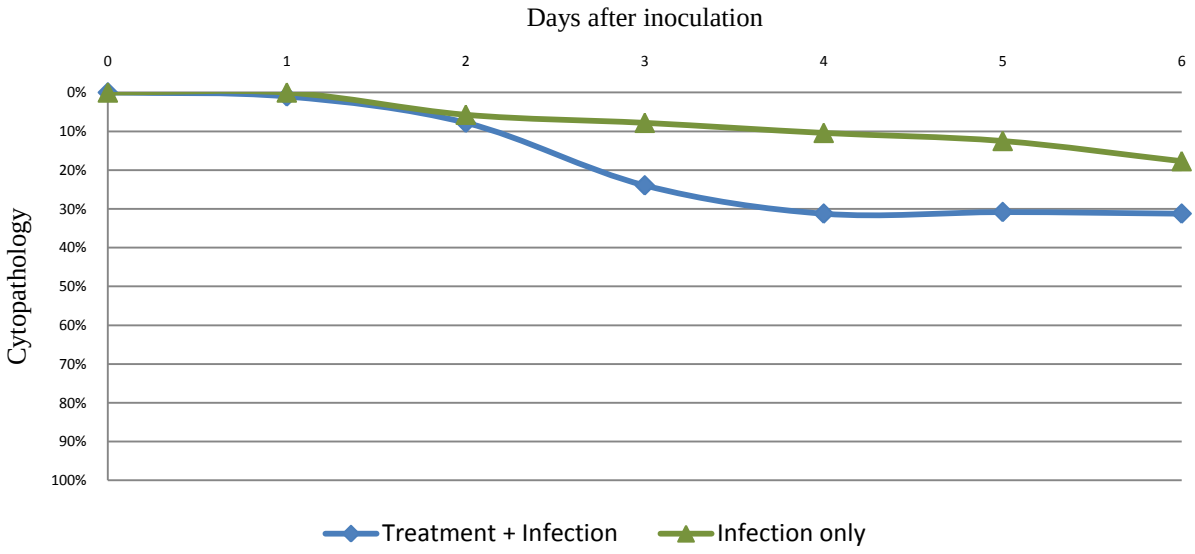
Graph 3-(a): level of viral cytopathology exhibited by A549 cells. Treatment + infection cells are experimental samples. Two control samples (treatment only, infection only) are also shown to observe whether the experimental samples exhibited a different level of cytopathology from the controls.



Graph 3-(b): level of viral cytopathology exhibited by A549 cells. Treatment + infection cells are experimental samples. Two control samples (treatment only, infection only) are also shown to observe whether the experimental samples exhibited a different level of cytopathology from the controls.



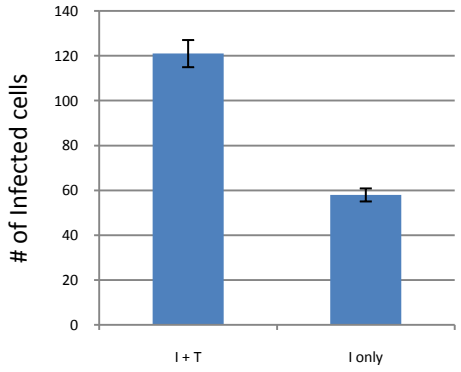
Graph 3-(c): level of viral cytopathology exhibited by A549 cells. Treatment + infection cells are experimental samples. Two controlled samples (treatment only, infection only) are also shown to observe whether the experimental samples exhibited a different level of cytopathology from the controls.



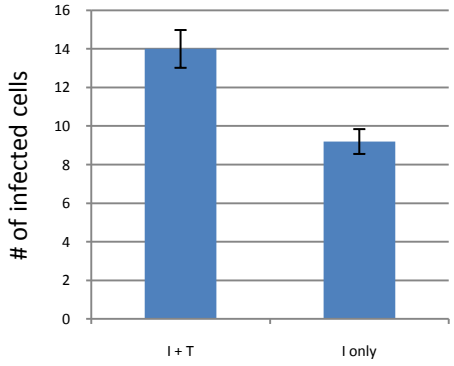
Graph 3-(d): level of cytopathology exhibited by A549 cells. Treatment + infection cells are the experimental samples. Compared to 3-(a), (b), and (c), only one controlled group (infection only) is shown.

Quantitative viral analysis was done in order to support the results of the qualitative analysis. Immunoassay is highly specific for the presence of viral antigens, thus using fluorophore-tagged secondary antibodies, which indirectly recognize BEV-1 viral proteins, to bind with infected cells would allow quantifying the numbers of infected cells based on fluorescent signals. Graphs 4-(a), (b), (c) show three different trials of immunofluorescent assay experiments; treated and infected A549 cells were compared to infected only cells to determine whether BEV-1 infectivity was different between the two. Result from 4-(a) shows significant difference between T+I cells and I only cells. T+I cells showed more than twice as many infected cells than I only cells. However, results from 4-(b) and (c) show fewer infected cells, and the difference between T+I and I only cells is not significant, especially for 4-(c) where the numbers of infected cells were nearly identical between T+I and I only cells.

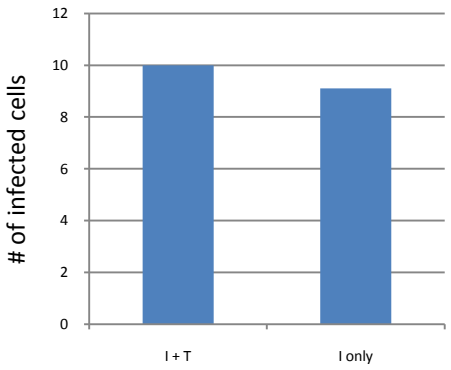
4-(a)



4-(b)



4-(c)



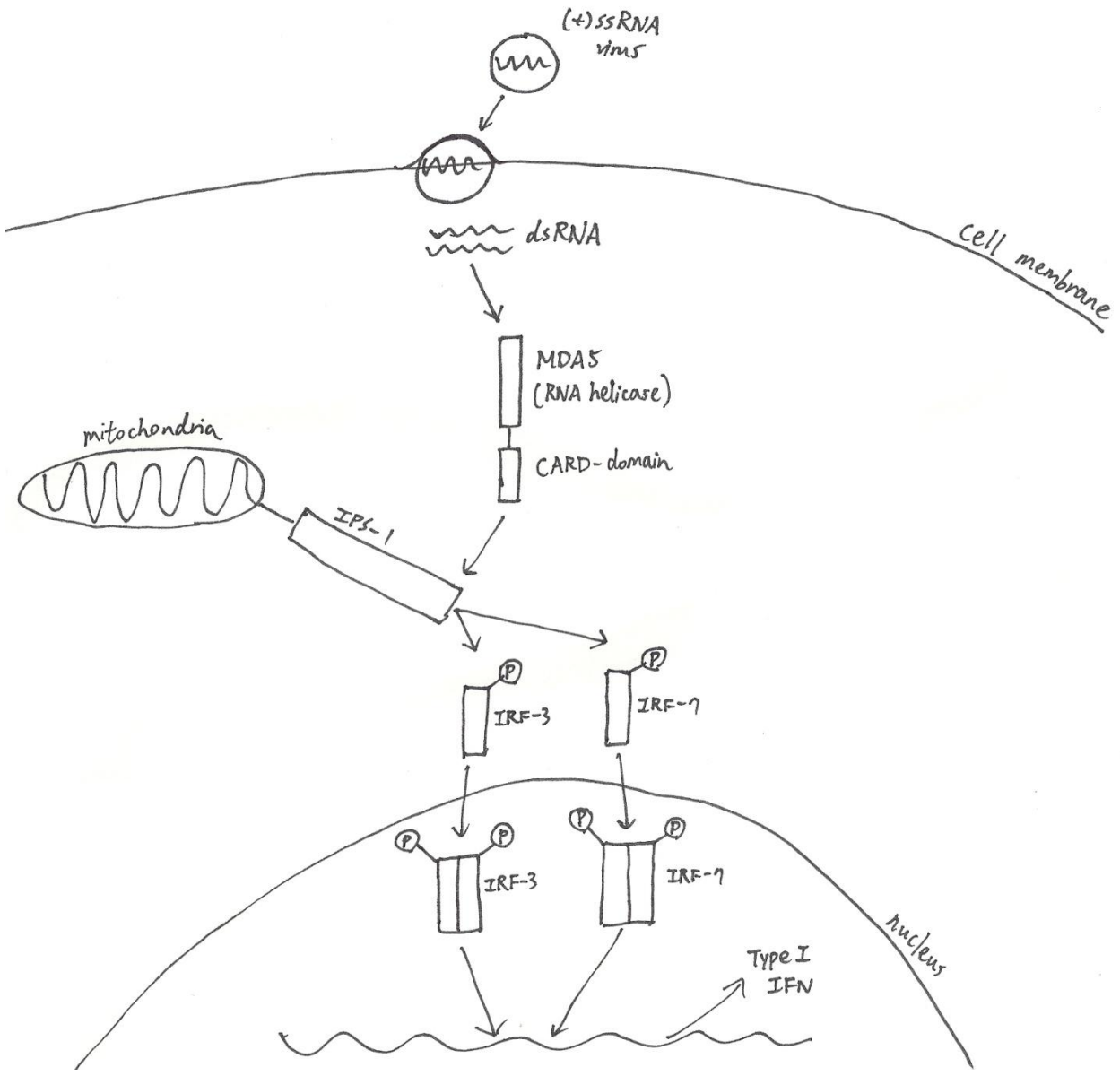
Graphs 4-(a), (b), and (c) show the numbers of infected cells for infected-and-treated (I+T) and infected-only (I only) A549 cells. Notice the scale of 4-(a) is nearly 10-fold higher than the scale used in 4-(b) and (c), showing that 4-(a) represents a more successful trial where higher numbers of cells were infected. 4-(c) does not contain error bars because only one sample of I+T and I only each are shown.

Discussions:

Qualitative analysis shows that fludarabine treatment enhances cytopathology of A549 cells when infected with BEV-1. By using controls such as drug-treatment-only and viral-infection-only, it is shown that the greater level of cytotoxicity is not a result of either viral infection or fludarabine toxicity alone, but rather a combined effect. On the contrary, quantitative analysis does not yield a definite conclusion supporting the hypothesis. Out of the three trials, only one trial yielded a more significant result which infected-and-treated cells showed higher viral yields than the infected-only cells, yet the other two trials did not show such difference. Since the approach of fludarabine treatment of cells for virology studies has not been done by other researchers, the procedure for this study, such as the host cell type, cell density, incubation time, fludarabine concentration, dose of BEV-1, or even the immunofluorescent assay, might not be suitable or ideal to yield an optimal result.

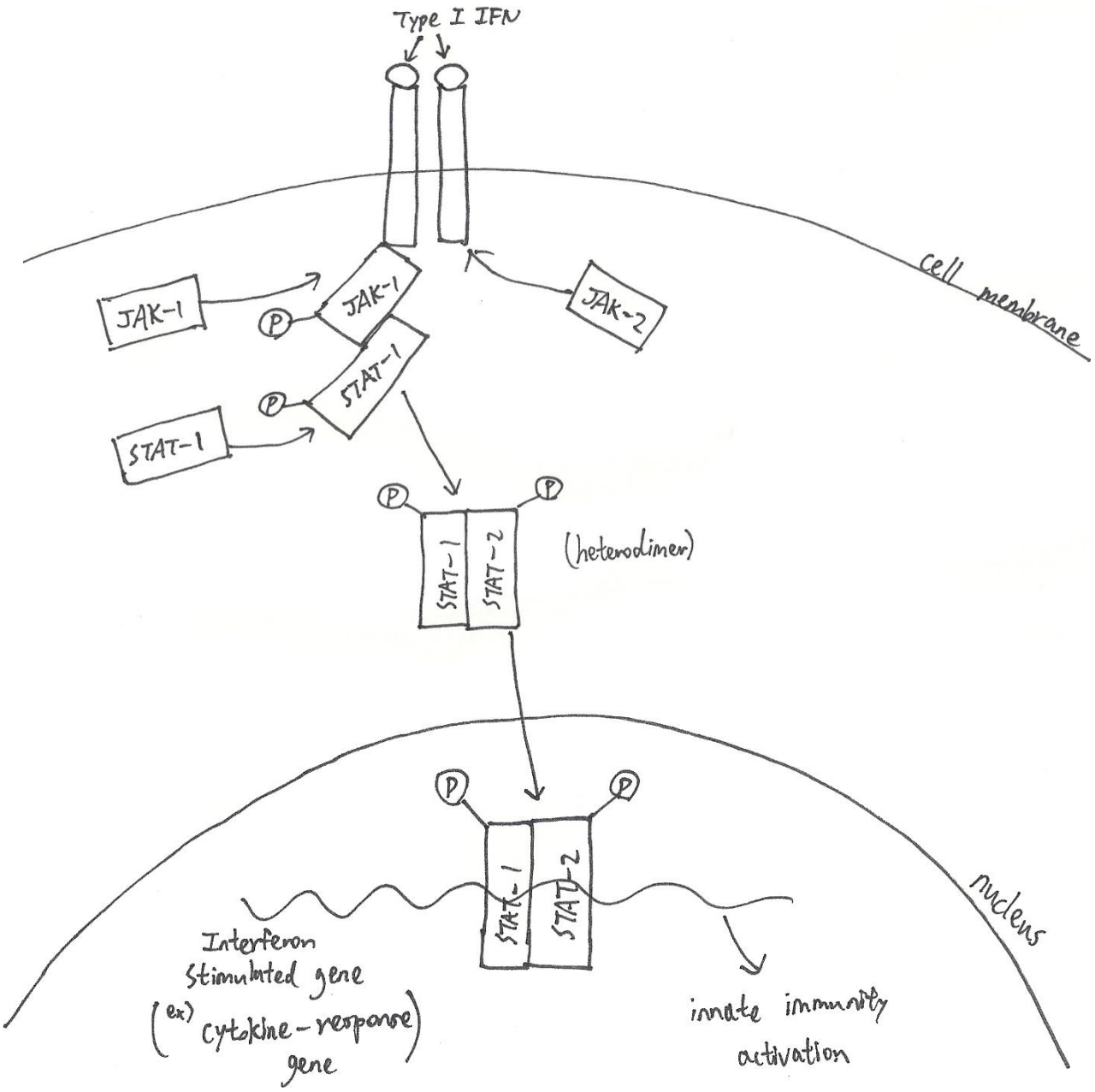
Using the results from qualitative and quantitative analyses which indicate enhancement in cytopathology and potentially in viral yield due to fludarabine, along with the studies that suggested how fludarabine interferes with components of innate immunity such as STAT-1, Graph 5-(c) is produced as a graphical representation of how fludarabine could affect a cell's innate immunity against a (+)ssRNA virus infection. Graphs 5-(a) and 5-(b), as mentioned in the introduction, depict the (+)ssRNA virus recognition, Type I IFN production, and up-regulation of innate immunity due to Type I IFN. Notice that it is still unclear whether fludarabine-treated cells are still able to recognize the virus but cannot induce anti-viral state due to STAT-1 suppression, or whether STAT-1 suppression results in inability of fludarabine-treated cells to recognize the virus due to MDA-5 suppression (or disruption of signal transducing pathway due to suppression of the intermediate proteins).

5-(a)



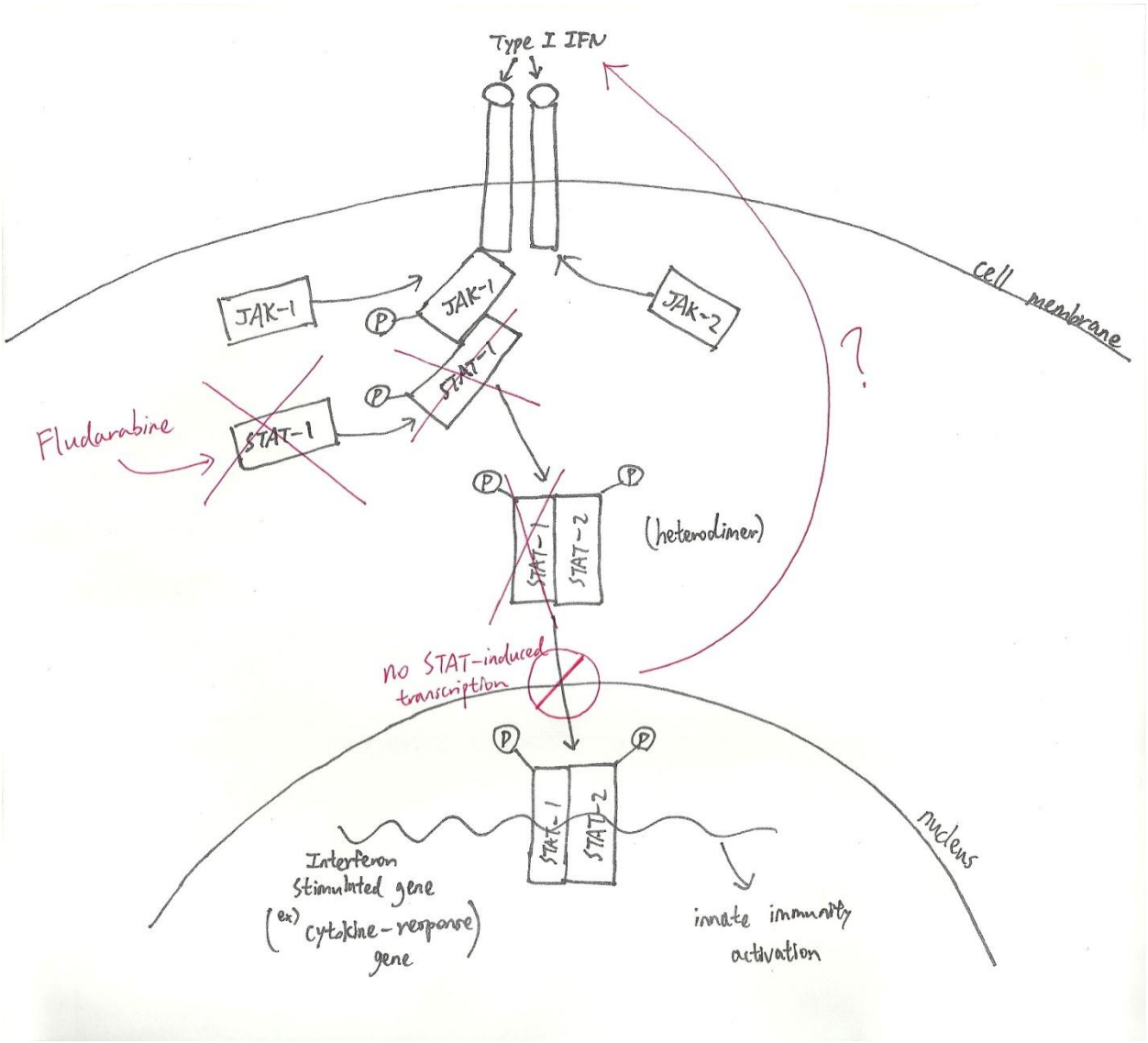
Graph 5-(a): a diagram showing the recognition of (+)ssRNA virus relies on MDA5, and the subsequent signal transductions which eventually leads to the transcription of Type I IFN encoded gene.

5-(b)



Graph 5-(b): a diagram showing the recognition of Type I IFN with the IFN receptors, and the signal transduction through STAT1-JAK pathway. The STAT-1/STAT-2 heterodimer leads to the transcription of interferon-stimulated gene, which then leads to the activation of innate immune responses, such as anti-viral state [3,19,22]. Notice that the graph does not show the phosphorylation of STAT-2; however, this process was similar to STAT-1 phosphorylation through JAK.

5-(c)



Graph 5-(c): Fludarabine's suppression on STAT-1 expression shuts down the STAT-1/JAK pathway, thus the subsequent heterdimer formation and attachment to the promoter region of interferon-stimulated gene are affected as well. It is still unknown whether the suppression on STAT-1 would only affect the activation of innate immunity, or if it would affect the Type I IFN production through some feedback inhibition mechanism.

While this study introduces a potential application of fludarabine on virology studies, further experimentations are needed in order to understand how exactly fludarabine is enhancing cytopathology and viral yields. For example, other experiments could further optimize the immunofluorescent viral detection methods or use alternative methods such as plaque assays. Also, one could compare fludarabine-treated cells to STAT-1 knocked-out cells and observe their susceptibility to BEV-1 / Norovirus infections; using genetically engineered cells could produce a possible answer whether fludarabine-treated cells are still able to recognize (+)ssRNA viruses, or the suppression of STAT-1 also influences MDA5 functions.

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